

Generation of human single-chain variable fragment antibodies specific to dengue virus non-structural protein 1 that interfere with the virus infectious cycle

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Abbreviations: ADE, antibody-dependent enhancement; ATCC, American Type Culture Collection; bp, base pair; BSA, bovine serum albumin; C, capsid; CMC, carboxymethyl cellulose; DAB, 3,3'-diaminobenzidine; DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; E, envelope; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Fc, crystallizable fragment; HRP, horseradish peroxidase; HuScFv, human single-chain variable fragment; Ig, immunoglobulin; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani; M, membrane; MEM, minimum essential medium; MOI, multiplicity of infection; NEAA, non-essential amino acid; NS1, non-structural protein 1; OD, optical density; PBS, phosphate-buffered saline; PBST, PBS with 0.1% (v/v) Tween-20; PCR, polymerase-chain reaction; PEG, polyethyleneglycol; pfu, plaque-forming unit; TMB, 3, 3', 5, 5', -tetramethylbenzidine; TPB, tryptose phosphate broth; V_H, variable region of the heavy chain; V_L, variable region of the light chain

Severe forms of dengue virus (DENV) infection frequently cause high case fatality rate. Currently, there is no effective vaccine against the infection. Clinical cases are given only palliative treatment as specific anti-DENV immunotherapy is not available and it is urgently required. In this study, human single-chain variable fragment (HuScFv) antibodies that bound specifically to the conserved non-structural protein-1 (NS1) of DENV and interfered with the virus replication cycle were produced by using phage display technology. Recombinant NS1 (rNS1) of DENV serotype 2 (DENV2) was used as antigen in phage bio-panning to select phage clones that displayed HuScFv from antibody phage display library. HuScFv from two phagemid transformed *E. coli* clones, i.e., clones 11 and 13, bound to the rNS1 as well as native NS1 in both secreted and intracellular forms. Culture fluids of the HuScFv11/HuScFv13 exposed DENV2 infected cells had significant reduction of the infectious viral particles, implying that the antibody fragments affected the virus morphogenesis or release. HuScFv epitope mapping by phage mimotope searching revealed that HuScFv11 bound to amino acids 1–14 of NS1, while the HuScFv13 bound to conformational epitope at the C-terminal portion of the NS1. Although the functions of the epitopes and the molecular mechanism of the HuScFv11 and HuScFv13 require further investigations, these small antibodies have high potential for development as anti-DENV biomolecules.

Introduction

Dengue virus (DENV) is the etiologic agent of dengue fever (DF) in humans, which is an important public health problem. The virus comprises four distinct serotypes (DENV 1–4).¹ The incidence of infection has increased more than 30-fold during the past three decades, and the endemic area of the

disease has expanded.^{2–4} The DENV infection is transmitted by *Aedes* mosquitoes, which have predilection for hot and humid climate. After the mosquito bite, the virus initially replicates in the skin and draining lymph nodes before becoming viremic.⁵ The incubation period of the disease ranges from 3–14 d. The outcome of DENV infection varies from mild fever to severe forms, i.e., dengue hemorrhagic fever (DHF) and dengue shock

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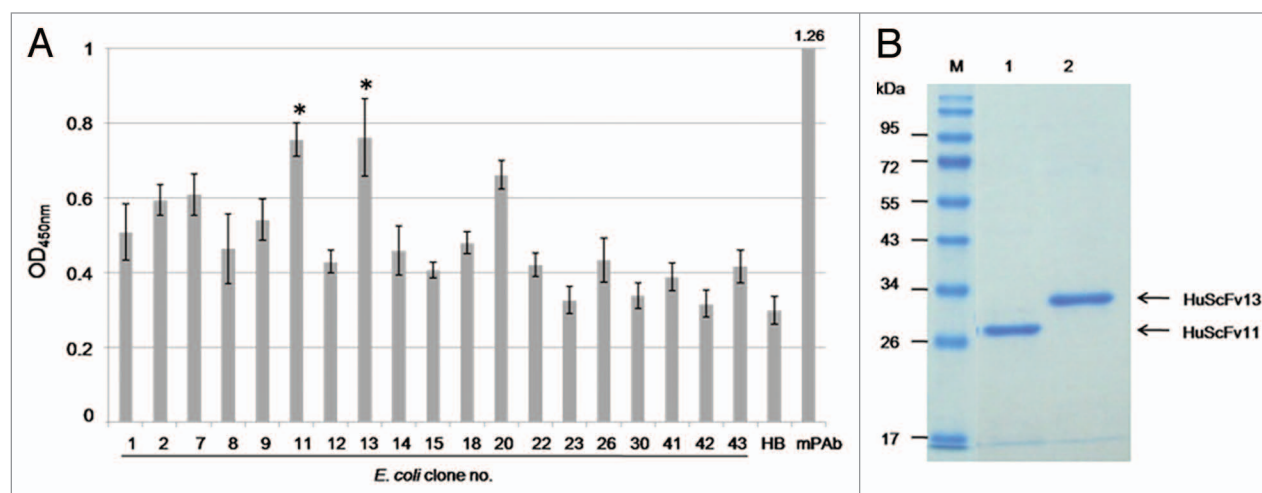


Figure 1. DENV2 NS1-specific HuScFv from bio-panning were testified and produced. **(A)** Indirect ELISA results for detecting the binding of HuScFv in lysates of 19 representative *huscfv*-phagemid transformed *E. coli* clones to native NS1. Clones no. 11 and 13 (asterisks) were selected for further use as the HuScFv in lysates of these clones gave the highest ELISA signal to the antigen compared with the signal from background (HB) control. Mouse anti-NS1 polyclonal antibody (mPAb) was used as positive control. Results are shown as OD_{450nm} of mean \pm SEM of three independent experiments. **(B)** Patterns of purified HuScFv11 and HuScFv13 were revealed by SDS-PAGE and CBB staining (lanes 1 and 2, respectively).

syndrome (DSS), that are characterized by vascular leakage, hemo-concentration, thrombocytopenia, coagulopathies, pleural effusion, hypovolumic shock, and organ failure.^{6,7} Severe dengue disease has high fatality rates if not treated promptly and adequately, especially in young children.⁸ There are several factors that predispose the DENV infection to DHF/DSS, one of which is the enhancement of intracellular replication of the heterotypic DENV in the secondary infection caused by non-neutralizing antibody sustained from the primary infection, so-called antibody dependent enhancement (ADE).⁹ Currently, there is no effective vaccine against DENV infection/disease. Avoidance of *Aedes* mosquito bites is the most practical intervention measure. Treatment of DHF/DSS cases is palliative. There is a pressing need of not only the DENV vaccine that can protect against multiple DENV serotypes of the viruses, but also a specific, effective and safe remedy for treatment of dengue disease.

During the DENV replication, ten functionally different viral proteins are produced, i.e., three structural proteins, including capsid (C), membrane (M), and envelope (E), and seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Among these proteins, NS1 (a 46-kDa glycoprotein containing 352 amino acids) is highly conserved across the four DENV serotypes. The protein exists in three functionally different forms, i.e., secreted hexameric, membrane bound homodimeric and intracellular monomeric. Although the biological activities of the NS1 are still elusive, evidence indicated that the secreted NS1 circulates in bloodstream and activates/modulates complement system and enhances DENV cellular entry.^{10,11} Plasma NS1 level correlated significantly with viremia and disease severity.¹² Homodimeric membranous NS1 interacts with lipid raft on the surface of infected cells, leading to initiation of intracellular signaling cascades and complement activation, which is a major pathophysiological finding in DHF/DSS cases.^{13,14} Intracellular monomeric NS1 was found to associate

with the viral RNA replication complex in the perinuclear region.¹⁵ DENV with NS1 mutation had impaired replication and virulence.^{16,17} Accumulated evidence indicates that the NS1 has a pivotal role in the DENV infectious cycle and contributes to disease severity. Thus, neutralization of the NS1 activities should lead to mitigation, if not abrogation, of the dengue disease severity.

DENV NS1 DNA vaccine-immunized animals develop both cellular and humoral immune responses to NS1 protein.¹⁸ Passive administration of NS1-specific antibodies has been shown to reduce viral load in DENV infected mice and primates.¹⁹ Thus, DENV NS1-specific antibodies are potential therapeutics against DENV infection. In the study presented here, human monoclonal single-chain antibody variable fragments (HuScFv) binding specifically to purified recombinant, secreted and intracellular native DENV2 NS1 were produced using phage display technology. The presence of HuScFv against DENV2 NS1 in culture medium could significantly reduce the amount of DENV2 released from the infected cells. The results of this study suggest that HuScFv might have utility as a specific and broadly effective anti-DENV biomolecule.

Results

Phage clones displaying NS1-specific HuScFv were selected by bio-panning

By using the purified rNS1 [from DENV serotype 2 (DENV2), strain 16681] as antigen in the phage bio-panning, 162 phagemid transformed HB2151 *E. coli* clones were selected, and 136 clones carried *huscfv* inserts (~1,000 bp). From 40 randomly selected *huscfv* positive clones, 28 clones could produce soluble HuScFv (26–34 kDa) as detected by western blot analysis using mouse anti-E tag as primary antibody. HuScFv in lysates of 19 transformed HB2151 *E. coli* clones were tested for binding

to secreted native NS1 of DENV2 by the indirect ELISA and HuScFv of all clones gave significant ELISA signals above the negative HuScFv control (lysate of original HB2151 *E. coli*) and control antigen (BSA) (Fig. 1A). HuScFv of two clones with the highest binding activities, i.e., clones no. 11 (HuScFv11) and 13 (HuScFv13), were selected for further study. After sub-cloning of the *huscfv* sequences from phagemids to pET23b⁺, affinity purified 6x His-tagged- HuScFv11 and HuScFv13 were prepared from the *huscfv*-pET23b⁺ transformed BL21 (DE3) *E. coli* (Fig. 1B).

HuScFv interacted to native NS1 in DENV-2-infected cells

Confocal microscopy (Fig. 2) revealed green fluorescence of native NS1 of DENV2 in cytoplasm, with high intensity in perinuclear region of the infected cells. The HuScFv11 and HuScFv13 (red fluorescence) were seen also in the cytoplasm. Yellow fluorescence in cytoplasm of the infected cells indicated co-localization of the HuScFv and the NS1. No fluorescent signal could be detected in MOCK-infected Huh-7 cell control stained similarly as the tests. Only green fluorescence of cytoplasmic NS1 protein was detected in DENV-2-infected cells incubated with irrelevant HuScFv control.

NS1 specific-HuScFv reduced DENV2 released from infected cells

Huh-7 cells were infected with DENV2 at MOI 0.1 or 1.0 and the cells were cultured in the presence of HuScFv11 or HuScFv13 for 24 and 36 h. Newly released DENV2 in the culture supernatants were determined by focus formation assay. Compared with the untreated control, HuScFv11 and HuScFv13 added to the culture medium at 12 h post-infection could significantly reduce the numbers of DENV2 released from the infected Huh-7 cells by at least 40% in all experimental conditions (Fig. 3A) ($P < 0.01$). The highest percent reduction (60%) was observed for the cells infected with DENV2 at MOI 0.1 and treated with HuScFv11 for 24 h. The amounts of the released virus from infected cells treated with irrelevant HuScFv and the infected cell controls were not different ($P > 0.05$).

The amounts of E antigen in the DENV2 infected cells (MOI 0.1 and 1.0) that were cultured in the medium containing either HuScFv11 or HuScFv13 were determined by cell ELISA in comparison with the amount of the antigen in DENV2 infected cells cultured in the medium alone. It was found that the OD_{405nm} of the cell ELISA of infected cells in all wells at 24 and 36 h post-treatment with HuScFv11 or HuScFv13 were not different statistically (Fig. 3B), implying that the HuScFv did not interfere with the DENV2 replication.

Phage mimotopes indicated presumptive NS1 peptides bound by HuScFv (HuScFv epitopes)

DNA of phage clones derived from panning of the Ph.D.-12TM peptide display phages with the immobilized HuScFv11 and HuScFv13 were individually extracted and sequenced. The amino acids of these phage clones were deduced. Peptide sequences of 17 and 16 clones could be obtained from HuScFv11 and HuScFv13 panning, respectively. The phage peptide sequences reacted with the HuScFv11 could be divided into 4 mimotope types, i.e., M11-1: VSVGAQRNATFT (5 clones), M11-2: VSVGAQRNATLR (4 clones) and 1 clone each of M11-3 to M11-9 (VSVGAQPECDVL,

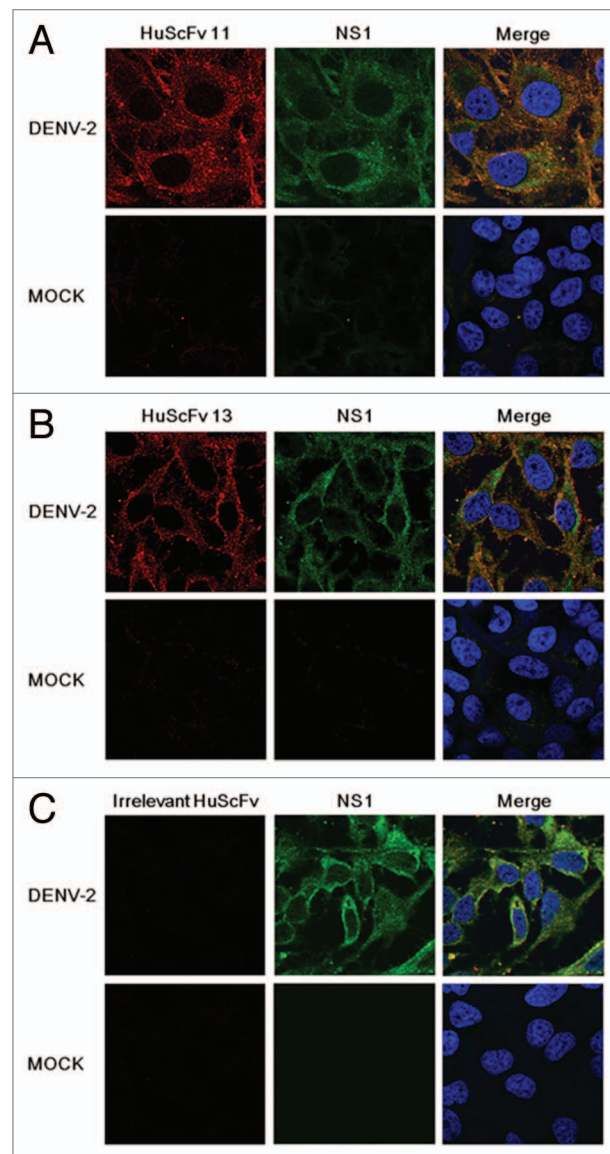


Figure 2. Immunofluorescent assay demonstrated interaction of HuScFv to native NS1 in DENV2-infected cells. Locations of HuScFv11 and HuScFv13 [red fluorescence in upper left panels of (A) and (B), respectively] and NS1 [green fluorescence in upper middle panels of (A), (B), and (C)] in Huh-7 cells infected with DENV2 revealed by confocal microscopy. After merging, the two proteins were found to co-localize [yellow fluorescence in upper right panels of (A) and (B)]. Cells stained with irrelevant HuScFv (C) and lower panels in (A), (B), and (C) are respective negative controls. Huh-7 nuclei are shown in blue fluorescence.

VSVGAHAECDV-, TAMMTNKHENCR, ASTYPQSSPGVT, WGNAYHRSALN, DYFNGSSAVHAA and ASVQGWAQITQR, respectively). There was only one mimotope type for HuScFv13, i.e., M13-1 (ATTTYWWTNISG). Mimotope consensus sequence of M11 mimotope types was VSVGAQRNAT. Pair-wise alignment of the HuScFv11 mimotope consensus sequence with DENV2 NS1 peptide sequence revealed that the mimotope matched with amino acid residues 1–14 (DSGCVVSWKNKELK) of the NS1 protein

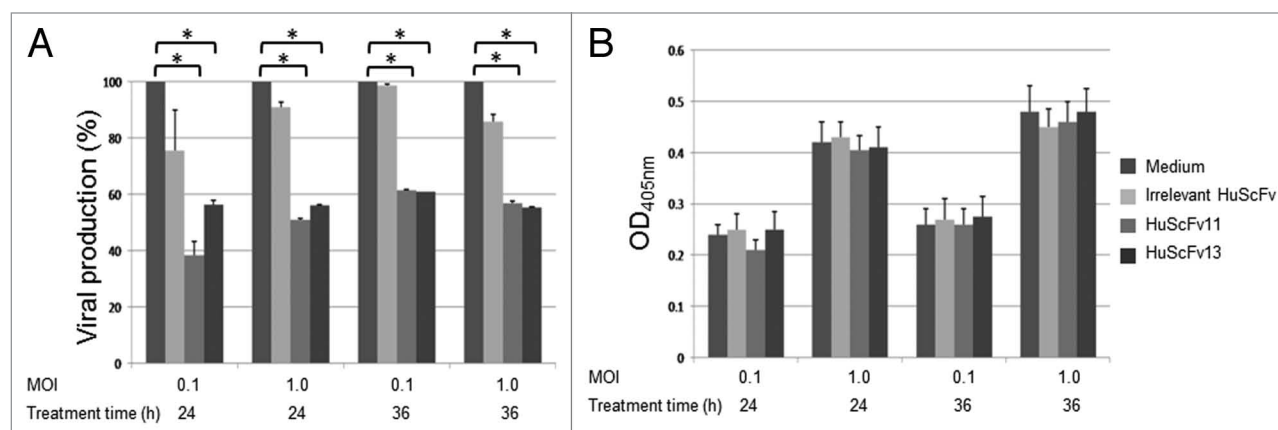


Figure 3. NS1 specific-HuScFv reduced DENV2 virus production from infected cells. **(A)** The HuScFv-exposed DENV-infected cells (MOI = 0.1 and 1.0) had markedly lower virus infectious particles in the culture supernatants at both 24 and 36 h post-treatment in comparison with the control (non-treated) cells. **(B)** Intracellular levels of the viral E protein in infected cells with or without HuScFv treatment determined by cell ELISA were not different. Data are mean + SEM of three independent experiments. *, significantly different from the controls at $P < 0.01$ by one-way ANOVA and Tukey's HSD test.

(Fig. 4A). The HuScFv13 mimotope (ATTTYWWTNISG) matched with amino acid residues in three regions throughout the C-terminal of the NS1, i.e., 164TTNIW168, 232WSN234 and 350VTA352 (Fig. 4B).

Pre-incubation of the synthetic NS1 peptides, i.e., DSGCVVSWKNKELK and TTNIWWSNVTA with HuScFv11 and HuScFv13 could reduce the HuScFv binding to NS1 in a dose-dependent manner (Fig. 4C). Irrelevant peptide control (peptide of loop-2 of *Naja kaouthia* long neurotoxin; WCDAFCSIRGKRV) pre-incubated with HuScFv did not reduce NS1 binding of both HuScFv11 and HuScFv13.

Discussion

The number of patients with severe DENV infection has increased markedly in many tropical and subtropical areas, including Southeast Asia, Africa, Middle East, Western Pacific and Americas.²⁰ There has been extensive research in laboratories around the world directed toward development of effective and safe DENV vaccine and anti-DENV agents. Presently, several vaccine candidates have reached Phase 3 trials, and a number of compounds and peptides with anti-viral potentials have been reported.^{21,22} At present, most DHF/DSS cases are given palliative treatment mainly in intensive care units.

NS1 is conserved across the four DENV serotypes, and has pivotal roles in the virus infectious cycles, including enhancement of viral entry into cells, replication, pathogenicity and mediation of pathophysiology.^{9-11,14,23,24} Although immune response to NS1 vaccine in interferon-deficient mice was limited, protective efficacy of anti-NS1 antibody after in vivo dengue virus or other flavivirus challenges has been demonstrated.²⁵⁻²⁹ The evidence indicated that NS1 is a potential vaccine target, and antibodies specific to this viral protein could be appropriate adjunctive remedy for passive immunotherapy of the diseases. However, activation of complement-mediated cytolysis by Fc-bearing anti-NS1 antibody may exacerbate the disease severity.³⁰ Thus, it was envisaged that a NS1-specific antibody suitable for dengue treatment should

be devoid of the Fc portion. Moreover, the antibody should be non-immunogenic and distributed rapidly within the recipient. HuScFv, which lack Fc and are approximately 5 times smaller (~25–35 kDa) than conventional IgG (~150 kDa), that bound specifically to, and interfere with, the functions of DENV NS1 conserved protein should be an appropriate format for dengue treatment.

In this study, HuScFv that bound specifically to DENV NS1 and inhibited the virus release from cells were produced. Monomeric recombinant NS1 (rNS1) was used as the antigen in bio-panning for selecting NS1 bound phage clones from a human ScFv phage display library.³¹ The sequences coding for the rNS1 (accession number JN692493) had 99% nucleotide similarity to the database sequence (accession number U87411.1). There were three mismatched nucleotides between the amplified cDNA of this study and the database sequences, i.e., 25G > A, 148T > C and 271C > T. Only the first mismatched position caused a change of arginine to lysine, but both are polar amino acids. Thus, the rNS1 produced in this study should retain all of the bioactivities of the native NS1.³² The HuScFv phage display library has been used successfully for production of HuScFv specific to other molecules, including *Naja kaouthia* neurotoxin, tetrodotoxin, and influenza virus proteins, i.e., polymerases, H5 hemagglutinin and matrix protein M1.^{20-23,33,34} As a time- and reagent-saving procedure, in this study one round of panning was performed instead of the multiple panning rounds reported by other laboratories.^{31,35} Among the phagemid-transformed *E. coli* clones, 84% carried the recombinant *huscFv*-phagemids, which conformed to the previous study showing that 85% of phages in this library carried *huscFv*.³¹ A fraction of the *huscFv*-phagemid *E. coli* clones (19 clones) were randomly selected for subsequent experiments while the other clones were kept for future requirement. All of the selected *huscFv*-positive clones derived from the panning with the monomeric rNS1 could express soluble HuScFv that bound to native DENV2 NS1 collected from the culture supernatant of infected cells, which was most likely to be hexameric forms, indicating that the

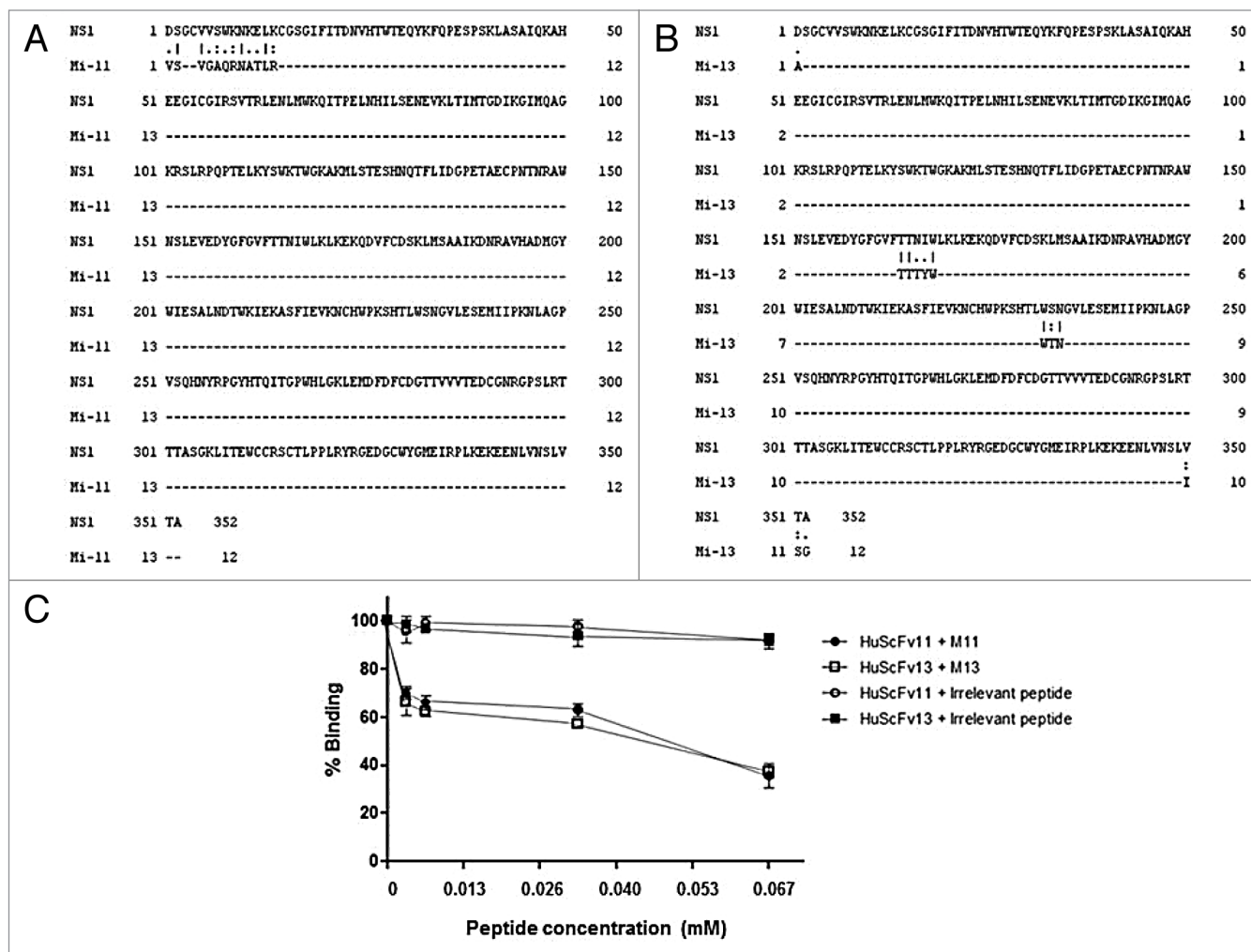


Figure 4. Mimotope sequences of HuScFv were aligned with the linear sequence of DENV2 NS1. (A) The tentative epitope of HuScFv11 (M11) located at the amino acid residues 1–14 (DSGCVVSWKNKELK) of NS1 protein sequence. (B) Amino acid sequence of the HuScFv13 epitope (M13) were residues 164–168 (TTNIW), 232–234 (WSN) and 350–352 (VTA) of NS1 protein sequence. A period indicates identical amino acids; a colon indicates conserved amino acid substitution; and an asterisk indicates semi-conserved amino acid substitution. (C) The synthetic peptides of epitopes of HuScFv11 (DSGCVVSWKNKELK) and HuScFv13 (TTNIWWSNVTA) could block the binding of the respective HuScFv to NS1 in the competitive ELISA in dose dependent manner while the irrelevant peptide control (WCDAFCSIRGKRV) did not affect the NS1 binding of HuScFv of both clones. Data are mean \pm SEM of three independent experiments.

HuScFv epitopes were maintained in the bacterially expressed monomeric NS1. The *huscfv* from the recombinant phagemid carried by two selected HB2151 *E. coli* transformants (clones no. 11 and 13) were subcloned into pET23b+ vector. The *huscfv*-pET23b+ transformed BL21 (DE3) *E. coli* produced relatively larger amounts of the HuScFv in the bacterial lysates than the *huscfv*-phagemid transformed HB2151 *E. coli* (data not shown) and the HuScFv still retained the binding activity to the rNS1 and to the native NS1 in the DENV2 infected cells. The finding that the HuScFv bound to both secreted and native NS1 led us to speculate that the HuScFv might be able to interfere with the NS1 multiple functions in the course of DENV infection, including interference of virus entry into cells and spread, which are the roles of secreted hexameric NS1, and interference with the complement modulation/activation by the membrane-anchored homodimeric NS1.^{10,11}

The culture supernatants of DENV2-infected Huh-7 cells grown in the medium containing either HuScFv11 or HuScFv13 for 24 and 36 h had significant reduction of the virus amounts compared with the culture fluids of the infected cells without HuScFv treatment. Less virus released into culture medium may be due to either less virus entry, replication, assembly or spread. The findings that the amounts of intracellular viral antigen in the HuScFv-exposed infected cells were not different from the control cells indicated that the NS1 specific-HuScFv might not interfere with the virus entry and intracellular replication, but inhibited the virus morphogenesis or release. Usually, the membrane lipid raft microdomain is a gateway for entry and release of several viruses.³⁶ Even though the HuScFv were unable to enter the cells, they might have posed at the virus exit and interfered with the virus release. It is also known that, during infection, DEN-infected cells have increased membrane

permeability.³⁷ Therefore, the diminutive HuScFv might have traversed the cellular membrane and bound intracellular NS1 which, in the effect, interfered with the virus morphogenesis. Experiments are needed to verify these speculations.

Mimotope/epitope mapping results indicated that the HuScFv might use either V_H or V_L, or both, for target binding. Although the functional significance of various NS1 regions and critical amino acid residues for the DENV infectious cycle are little known and require further elucidation, the data of this study indicated that the NS1 sites bound by the HuScFv11 (amino acids 1–14) and HuScFv13 (conformational epitope in the C-terminal) led to interference of the virus replication cycle by reducing DENV2 release into the infected cell milieu. To our knowledge, this is the first annotated report on fully human ScFv that target NS1. The HuScFv have high potential for further development into adjunctive, if not sole, anti-DENV biomolecules for DENV infection.

Materials and Methods

Dengue virus

DENV serotype 2 (DENV2), strain 16681, were propagated in *Aedes albopictus* C6/36 cell line (ATCC CRL-1660) grown in Leibovitz's (L-15) medium supplemented with 10% (v/v) heat-inactivated FBS (Gibco), 10% (v/v) TPB and 1% (v/v) penicillin G and streptomycin at 28 °C. The amount of DENV2 in the culture supernatant was titrated by focus formation assay and the virus was kept at -80 °C until use.

Cell cultures

African green monkey kidney (Vero) cells (ATCC CRL-1651) grown in Minimum Essential Medium (MEM) (Gibco) containing 10% (v/v) FBS, 2 mM L-glutamine, and 1% (v/v) antibiotics at 37 °C in 5% CO₂ atmosphere were used in the focus formation assay for DENV titration. Human hepatocellular carcinoma (Huh-7) cells (JCRB0403) grown in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) antibiotics and 1% (v/v) NEAA (complete RPMI medium) at 37 °C in 5% CO₂ atmosphere were used in an assay for determining the activity of the NS1 specific-HuScFv on DENV replication cycle.

Preparation of recombinant NS1 (rNS1)

Viral RNA was extracted from culture supernatant of DENV2 infected C6/36 cells by using QIAamp MinElute Virus Spin Kit (Qiagen) and reverse transcribed to cDNA by using NS1-R primer (5'-CCG CTC GAG AGC TGT GAC CAA GGA GTT GAC CAA A-3') and SuperScriptTMIII First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was used as a template for amplification of full-length NS1-coding sequence by Platinum[®] Pfx DNA polymerase (Invitrogen). The PCR primers were: NS1-F (5'-CCC GGA TCC GAT AGT GGT TGC GTT GTG AGC TGG A-3') and the NS1-R primer. BamHI and XhoI endonuclease sites (underlined) were incorporated into the primer sequences to facilitate subsequent DNA cloning. PCR amplicon was detected by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining. The NS1-coding sequence was ligated with pET21b+ via the endonuclease restriction sites and

the recombinant plasmid was introduced into BL21 (DE3) *E. coli*. The DNA sequence coding for full-length DENV serotype 2 (DENV2) NS1 (~1,200 bp) (accession number JN692493) had 99% sequence similarity to the representative DENV NS1-coding sequence in the NCBI database (accession number U87411.1). A colony of transformed *E. coli* grown on LB agar plate containing 100 µg/ml Ampicillin (LB-A) was selected for production of recombinant NS1 (rNS1) protein (~43 kDa) under IPTG induction. The 6 × His-tagged rNS1 in the bacterial lysate was detected by western blot analysis. The rNS1 was affinity purified from the bacterial lysate by using TALONTM Metal Affinity Resin (Clontech). The resin-bound protein was eluted by using gradient solutions of imidazole. Fractions containing purified rNS1 were determined by 12% SDS-PAGE and Coomassie brilliant blue G-250 (CBB) (Sigma-Aldrich) staining. Purified rNS1 was dialyzed against 0.01 M PBS, pH 7.4 and kept at -20 °C until use.

Phage bio-panning

The purified rNS1 was used as antigen in the phage panning for selecting phage clones that displayed rNS1 bound HuScFv from a HuScFv phage display library constructed previously.^{31,38} Briefly, 100 µl of rNS1 (1 µg) in coating buffer (carbonate-bicarbonate buffer, pH 9.6) were added to a well of E.I.A./R.I.A. Strip (Costar[®]) and kept at 37 °C for 16 h. Excess protein was discarded and the well was washed with PBS and blocked with 3% skim milk in PBS. The HuScFv phage display library (100 µl containing ~10¹¹ pfu) was added to the antigen-coated well and incubated. After excessive washing to remove unbound phages, 100 µl of log phase grown HB2151 *E. coli* were added to the well containing rNS1-bound phages to allow phage transduction. The phagemid-transformed bacterial clones were selected on LB-A agar plate containing 2% glucose (LB-AG). *E. coli* colonies were randomly picked from the overnight agar plate and screened for the presence of HuScFv coding sequence (*hscfv*) by PCR using phagemid-specific primers, i.e., R1 (5'-CCA TGA TTA CGC CAA GCT TTG GAG CC-3') and R2 (5'-CGA TCT AAA GTT TTG TCG TCT TTC C-3'). The *hscfv* positive clones were grown individually under 1 mM IPTG induction in LB broth. HuScFv in the bacterial lysates were detected by western blot analysis using rabbit anti-E tag polyclonal antibody (Abcam), swine anti-rabbit IgG-HRP-conjugate (Dako), and Super Signal[®] West Pico chemiluminescent substrate (Thermo Scientific). HuScFv in the *E. coli* lysates were standardized by using spectrophotometer.

Indirect ELISA

To determine antigenic specificity of the HuScFv to NS1, culture medium of DENV2-infected Vero cells containing secreted NS1 was precipitated with 50% saturation of ammonium sulfate and dialyzed against PBS. Ten µg of either the native NS1 preparation (antigen) or culture medium of the non-infected Vero cells (negative antigen control) in 100 µl of coating buffer were added to separate wells of a Maxisorb Immunoplate (Nunc) and the plate was kept at 37 °C overnight. Excess fluid was discarded and the wells were washed with PBST, *E. coli* lysates containing HuScFv (1 mg in 100 µl) were added individually to the negative antigen control wells, incubated at 37 °C for 1 h

before transferring the fluids to the native NS1-coated wells and incubated at 37 °C for 2 h. The wells were washed with PBST and the NS1 bound HuScFv were detected by rabbit anti-E tag polyclonal antibody followed by swine anti-rabbit IgG-HRP-conjugate and TMB substrate (Invitrogen). The reaction was stopped by adding 3 M H₂SO₄. Optical density at 450 nm (OD_{450nm}) of the content in each well was determined by using an ELISA reader (MultiskanEX, Thermo Scientific). Lysate of original HB2151 *E. coli* host cells and mouse polyclonal antibody to recombinant NS1 (mPAb) served as background and positive antibody controls, respectively.

HuScFv preparation

For large scale production of purified HuScFv, *huscfv* sequences from selected *E. coli* clones were subcloned into pET23b+ vector and the *huscfv*-pET23b+ vectors were introduced into BL21 (DE3) *E. coli*.³⁹ Selected *E. coli* transformants were grown under 0.5 mM IPTG induction at 37 °C for 3 h and the 6x His-tagged-HuScFv were purified from individual bacterial lysates by using affinity resin.

Indirect immunofluorescent assay

Huh-7 cells (5 × 10⁴ cells/well) were grown on glass coverslips placed in wells of 24-well tissue culture plate for 24 h. The cells were rinsed with RPMI medium and incubated with DENV2 at MOI 0.1. Cells added with complete RPMI medium served as non-infected control. After incubating at 37 °C in 5% CO₂ atmosphere for 2 h, the supernatant was removed; the cells were rinsed, replenished with complete RPMI medium and incubated as above for 3 d. The DENV2 infected cells and the MOCK were fixed with 3.6% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, blocked with 3% BSA in PBS and incubated with purified HuScFv (2.5 μM) at 37 °C for 2 h in a humidified chamber. Thereafter, the fixed cells were rinsed with PBS and incubated with a mixture of rabbit polyclonal antibody to human single-chain antibody fragments and mouse anti-NS1 (both diluted 1:1000) at 37 °C for 2 h. After washing, the cells were incubated with a mixture of CyTM3-conjugated AffiniPure donkey anti-rabbit Ig (JIR Laboratories), Alexa Fluor 488-conjugated goat anti-mouse Ig (Molecular Probe), and anti-nuclear staining reagent (Hoechst; Molecular Probe) (diluted 1:1,000) to localize HuScFv, NS1, and nuclear DNA, respectively. The mounted fluoresced cells were observed under laser scanning confocal microscope (LSM 510 META, Carl Zeiss).

DENV infection reduction assay

Huh-7 cells (1 × 10⁴ cells/well) were cultured in complete RPMI medium in 96-well tissue culture plate for 24 h. The cells were rinsed twice with plain RPMI medium before DENV2 at MOI 0.1 or 1.0 (50 μl/well) was added and incubated at 37 °C for 2 h. Fluid in each well was discarded, the cells were rinsed, 150 μl of complete RPMI medium were added to each well and the cells were cultured for 12 h. Culture supernatant was then removed, the cells were rinsed twice with the RPMI medium, replenished with the medium containing either NS1 specific-HuScFv (2.5 μM) or irrelevant HuScFv (2.5 μM) or the medium alone (six wells for each treatment) and incubated at 37 °C, 5% CO₂ atmosphere. Culture supernatants were collected

from three wells per treatment at 24 and 36 h post-treatment. The numbers of DENV2 in the supernatants were determined by using focus formation assay. Percentage of DENV production was calculated as (foci from culture supernatant with treatment/ foci from untreated culture supernatant) × 100%. Infected cells in the wells were subjected to cell ELISA for semi-quantification of intracellular DENV envelope (E) antigen.⁴⁰

Focus formation assay

The assay was performed as described previously with modifications.⁴¹ Briefly, each culture supernatant was diluted 10-fold serially in MEM and 50 μl of each dilution were added appropriately to Vero cell monolayer in 96-well tissue culture plate. After allowing virus absorption at 37 °C for 2 h, the cells were rinsed and cultured in 200 μl of MEM supplemented with 1% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) antibiotics and 2% (v/v) CMC at 37 °C for 3 d. The culture fluids were discarded and the cells were rinsed before fixing with 3.6% formaldehyde and permeabilized with 1% Triton X-100, added with mouse monoclonal anti-DENV E protein antibody of clone 4G2 (ATCC) and incubated at 37 °C for 2 h. After washing, rabbit anti-mouse Ig-HRP conjugate (Dako) and DAB substrate (Sigma-Aldrich) were used as focus revelation reagents. The foci were enumerated under a light microscopy.

Cell ELISA

The assay was used for determining the amount of intracellular DENV E antigen. The infected Huh-7 cells, either treated with HuScFv or controls, in the wells were washed, fixed with formaldehyde, permeabilized with 1% (v/v) Triton X-100 in PBS, blocked with FBS, and added with mouse monoclonal anti-DENV E protein antibody (clone 4G2, ATCC). Color development was achieved by adding rabbit anti-mouse Ig-HRP conjugate and ABTS[®] peroxidase substrate (KPL). The fluid in each well was appropriately placed in a new well of a microplate and OD_{405nm} was determined against blank (non-infected cells cultured similarly as the infected cells).

HuScFv epitope searching

Phage clones displaying peptides that bound to the NS1-specific HuScFv (phage mimotopes) were selected from Ph.D.-12TM Peptide Library (New England Biolab) as described previously.³⁵ Briefly, purified NS1-specific HuScFv (10 μg) was coated onto surface of microtiter well. After blocking the well with 10% skim milk, 100 μl of the phage library were added and the plate was placed on a rocking platform at 25 °C for 1 h. Unbound phages were removed, the well was washed and added with 150 μl of mid log phase grown ER2738 *E. coli*, kept at 25 °C for 20 min and the phage transfected *E. coli* were grown in LB broth at 37 °C for 4.5 h. Propagated phages were collected by precipitating the bacterial culture supernatant with PEG-NaCl and subjected to the next panning round. The panning was repeated two more times and the phages collected from the *E. coli* culture supernatants of the third panning round were plated appropriately on agarose topped-LB plate containing X-Gal and IPTG. Phage DNA was extracted from randomly picked blue plaques and sequenced. The 12-mer peptide insert of each phage clone was deduced. Consensus mimotope sequence was obtained by multiple alignments of the deduced

peptide sequences (EMBOSS ClustalW2). The mimotope consensus sequences were aligned (EMBOSS Needle) with the NS1 amino acid sequence (accession number U87411.1) and the NS1 peptides matched with the mimotope consensus sequences (HuScFv epitopes) were determined.

Competitive ELISA

Phage mimotope searching results were verified by using peptide competitive ELISA. Commercially-synthesized peptides of the NS1 matched with the phage mimotope consensus sequences or irrelevant peptide control (peptide of loop-2 of *Naja kaouthia* long neurotoxin) were used as competitors of the HuScFv binding to the immobilized NS1 in the ELISA wells. Various concentrations of the synthesized peptides were mixed with 5 μ M of purified His-tagged-HuScFv and incubated at 37 °C for 2 h prior to adding the mixtures to wells coated with native NS1 (10 μ g per well). After incubating at 37 °C for 2 h, the amounts of the NS1-bound HuScFv in all wells were detected by using mouse anti-6x His antibody followed by rabbit anti-mouse Ig-HRP-conjugate (Dako). TMB substrate and 3 M H₂SO₄ were added before measuring OD_{450nm} of all well contents. The OD_{450nm} in NS1-coated wells added with the respective HuScFv without pre-incubation with the peptides were used as 100% binding controls. Percent HuScFv binding to the NS1 was calculated.

Statistical analysis

The numbers of the DENV2 released from Huh-7 infected cells and the result of cell ELISA E antigen in the infected cells treated with HuScFv and negative HuScFv control (results from three independent experiments) were analyzed and compared statistically by one-way ANOVA and Tukey's HSD test. Significant difference was $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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